Arch Toxicol (1990) 64: 188–192

Archives of
Toxicology

Mono-*ortho*-chlorinated chlorobiphenyls: toxicity and induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity in chick embryos

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Abstract. Six mono-*ortho*-chlorinated chlorobiphenyls were compared regarding their toxicity and 7-ethoxyresorufin *O*-deethylase (EROD)-inducing potency in chick embryos. Three of the tested chlorobiphenyls have a chloro substituent adjacent to the *ortho*-chlorine, and these congeners were about ten times more potent than the three having a *meta*-hydrogen adjacent to the *ortho*-chlorine. These more toxic mono-*ortho*-chlorinated congeners were, however, about three orders of magnitude less toxic and less potent as EROD inducers in chick embryos than 3,3′,4,4′,5-pentachlorobiphenyl in a previous similar study. Malformed eyes and beaks, degenerative hepatic lesions and subcutaneous as well as pericardial edema were detected in embryos exposed to the mono-*ortho*-chlorine-substituted congeners, as was previously found after exposure to the most toxic non-*ortho*-chlorinated, coplanar chlorobiphenyls. It is concluded that the mono-*ortho*-chlorinated chlorobiphenyls are considerably less toxic and less potent as EROD inducers than the most toxic coplanar ones. Owing to their relatively high concentrations in technical preparations of polychlorinated biphenyls (PCBs) the mono-*ortho*-chlorine-substituted congeners may, however, contribute to the overall toxicity of PCBs.

Key words: Chick embryos – Environmental pollutants – Enzyme induction – Polychlorinated biphenyls – Toxicity

Introduction

Theoretically, 209 chlorobiphenyls can exist, differing in their number and positions of chlorine atoms, and around 100 of these are found in technical preparations of polychlorinated biphenyls (PCBs) (Albro et al. 1981). In recent years much interest has been focused on the toxicity of individual chlorobiphenyls in the complex technical PCB mixtures. The non-*ortho*-chlorinated PCBs can assume a coplanar conformation, and it is among these congeners that the most toxic ones are found. The most potent of the coplanar chlorobiphenyls are the ones chlorinated in the *para* positions and in at least one *meta* position of each ring. These congeners, i.e. 3,3′,4,4′-tetraCB, 3,3′,4,4′,5-pentaCB and 3,3′,4,4′,5,5′-hexaCB, are, in their coplanar conformations, approximate isostereomers of 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD). They bind to the *Ah* receptor and have biological effects similar to those of TCDD (Safe 1984). The presence of these toxic coplanar chlorobiphenyls in technical PCB preparations as well as in environmental and human samples further emphasizes the environmental hazards posed by these congeners (Kannan et al. 1987; Tanabe et al. 1987).

The chlorobiphenyls chlorinated in one of the *ortho* positions also bind to the *Ah* receptor, although less avidly than the most toxic coplanar congeners (Poland and Glover 1977; Bandiera et al. 1982). The mono-*ortho*-chlorinated chlorobiphenyls, however, have been reported as being present in considerably higher concentrations in some technical PCBs than the non-*ortho*-chlorinated ones (Kannan et al. 1988), and may thus contribute substantially to the toxicity of the technical preparations.

Chick embryos have proved to be extremely sensitive to dioxins and related compounds. In addition to embryolethality, these substances cause edema and malformations such as microphthalmia and shortened beak (Firestone 1973; Schrankel et al. 1982; Brunström and Darnerud 1983). They also induce certain cytochrome P-450-dependent enzyme activities in the embryos, e.g. aryl hydrocyrbon (benzo[α]pyrene) hydroxylase (AHH) and 7-ethoxyresorufin *O*-deethylase (EROD) (Brunström 1986; Brunström and Andersson 1988).

In the present study, six mono-*ortho*-chlorinated PCBs were compared regarding their toxicity and EROD-induc-

ing potency in chick embryos. The results are compared with data from a previous study on coplanar, non-*ortho*-chlorinated PCBs.

Materials and methods

Eggs. Hens' eggs (White Leghorn, Shaver) were obtained from Linköpings Kontrollhönseri, Linköping, Sweden. They were incubated in a forced-draught incubator maintained at 60% relative humidity and 37.5–38.0° C, and they were turned every 6 h.

Chemicals. The mono-*ortho*-chlorinated chlorobiphenyls tested (IUPAC number in brackets) were 2,3′,4′,5-tetrachlorobiphenyl (70), 2,3,3′,4,4′-pentachlorobiphenyl (105), 2,3′,4,4′,5-pentachlorobiphenyl (118), 2,3,3′,4,4′,5-hexachlorobiphenyl (156), 2,3,3′,4,4′,5′-hexachlorobiphenyl (157) and 2,3′,4,4′,5,5′-hexachlorobiphenyl (167), all of which were synthesized by Dr Åke Bergman, Wallenberg Laboratory, Stockholm University, Sweden.

7-Ethoxyresorufin, bovine serum albumin (fraction V), NADPH and rhodamine B were purchased from Sigma (St Louis, Mo., USA) and resorufin from Eastman Kodak Co. (Rochester, NY, USA).

Toxicity studies. Two experimental procedures were used for evaluating the toxicity of the mono-*ortho*-chlorinated PCBs in chick embryos.

In the first procedure, the substances were injected into the air sacs of eggs preincubated for 7 days, and the eggs were further incubated for 72 h. Before injection the eggs were candled, and infertile eggs as well as those containing dead or poorly developed embryos were discarded. In preliminary experiments it was found that three of the chlorobiphenyls were considerably more toxic than the other three. Five different doses (0.25–4 mg/kg) of these three more potent congeners were injected for determination of their 72-h LD_{50} values, whereas a single dose (4 mg/kg) of the less toxic ones was tested. The substances were dissolved in peanut oil and injected through holes drilled in the shells (50 µl oil/egg, 20 eggs/group). Immediately after injection, the eggs were rotated to evenly distribute the oil and thereafter the holes were sealed with paraffin. The eggs were placed in the incubator with the blunt end upwards for 1 h and were then turned horizontally. Seventy-two hours after injection the eggs were candled to determine mortality rates.

In the second test, the substances were dissolved in an emulsion of peanut oil, lecithin and water and injected into the yolks of eggs preincubated for 4 days (100 µl emulsion/egg, 20 eggs/dose) as previously described (Brunström and Darnerud 1983). The three more toxic congeners were injected at three different doses (0.1, 0.5 and 2.5 mg/kg) whereas the less toxic ones were administered at a single dose (5 mg/kg). The eggs were candled every 2–3 days until the end of the experiment, 2 weeks after injection (day 18). Eggs containing dead embryos were opened and the embryos grossly examined for edema and malformations. At the end of the experiment all embryos were inspected and the occurrence of abnormalities noted.

Induction of EROD activity. Eggs were injected on day 7 of incubation using the air-sac route as described above, and EROD activities in the livers were determined 72 h later. The highest dose of each substance was lower than the corresponding LD_{50} in the 72-h toxicity test. Each liver was homogenized in a Potter-Elvehjem homogenizer (glass/Teflon) in 350 µl (controls) or 1400 µl (treated) of 150 mM Tris-HCl buffer. The EROD activity determinations were performed in duplicate using fresh homogenate.

EROD activity was determined essentially as described by Pohl and Fouts (1980). The reaction mixture (pH 7.8) contained 0.36 µmol NADPH, 3.0 µmol $MgCl_2$, 1.6 mg bovine serum albumin, 100 µmol Tris-HCl, 0.1 ml liver homogenate and 1 nmol 7-ethoxyresorufin in a total volume of 1.0 ml. The amount of tissue in the reaction mixture ranged from 2 to 12 mg. The reaction times were 10 min for the controls and 2 or 5 min for the induced livers, and the reaction temperature was 37° C. Under the conditions employed, the amount of product formed was proportional to the amount of tissue and to the reaction time. Reactions were stopped by adding 2.5 ml methanol, and the tubes were left at 37° C in the water-bath for 10 min to facilitate precipitation of the proteins. The tubes were centrifuged, and the fluorescence of the supernatants was determined at an excitation wavelenght of 530 nm and an emission wavelength of 585 nm. A solution of rhodamine B in ethylene glycol was used as a daily standard. This solution had previously been calibrated against resorufin.

Statistics. LD_{50} values were estimated by probit analysis of the mortality data using the Statistical Analysis System and the PROBIT procedure (SAS Institute Inc., Cary, NC, USA). Frequencies of mortality after treatment with the various chlorobiphenyls were compared with control values using the Chi square test of heterogeneity. However, when the smallest expected frequency was less than five, the Fisher exact probability test (Fisher 1934) was used.

Results

Of the six mono-*ortho*-chlorinated PCBs tested in the present study the three having the *ortho*-chlorine adjacent to a *meta*-chlorine were more toxic to the chick embryos than the three having the *ortho*-chlorine adjacent to a *meta*-hydrogen. The LD_{50} values determined in the 72-h toxicity test for the three most toxic congeners are presented in Table 1. 2,3,3′,4,4′,5-HexaCB had the lowest LD_{50} value [1.5 mg(4.2 µmol)/kg] whereas the LD_{50} values for 2,3,3′,4,4′-pentaCB and 2,3,3′,4,4′,5′-hexaCB were similar [2.2 mg(6.7 µmol) and 2.5 mg(6.9 µmol)/kg, respectively]. The three less toxic congeners were injected using a single dose of 4 mg/kg egg without causing any significant increase in embryonic mortality (Table 1).

The congeners found to be most potent in the 72-h study were also most potent in the 2-week toxicity test (Table 2). Almost all embryos (17–19/20) treated with the highest dose (2.5 mg/kg egg) of these more toxic congeners died before the end of the experiment (day 18). A 5-fold lower dose of these congeners also resulted in significant increases in embryolethality. Many of the embryos surviving the two highest doses exhibited microphthalmia; shortened beak, degenerative hepatic lesions and/or edema (subcutaneous, pericardial). At the lowest dose administered (0.1 mg/kg) no effects on the survival

Table 1. LD_{50} values and their 95% confidence limits for three mono-*ortho*-chlorinated chlorobiphenyls in chick embryos. The compounds were injected into the air sacs of eggs preincubated for 7 days, and mortality rates were determined 72 h later. Single doses of three less potent mono-*ortho*-chlorinated congeners were similarly injected, and the mortality rates are shown

Substance	LD_{50} (mg (µmol)/kg egg)	95% Confidence limits
2,3,3′,4,4′-pentaCB	2.2 (6.7)	1.9 (5.8)–2.5 (7.7)
2,3,3′,4,4′,5′-hexaCB	2.5 (6.9)	2.1 (5.8)–2.9 (8.0)
2,3,3′,4,4′,5-hexaCB	1.5 (4.2)	1.2 (3.3)–1.9 (5.3)

Substance	Dose (mg/kg egg)	Embryonic mortality	
		Ratio	%
Vehicle (peanut oil)	–	0/20	0
2,3′,4′,5-tetraCB	4	4/20	20[a]
2,3′,4,4′,5-pentaCB	4	2/20	10[a]
2,3′,4,4′,5,5′-hexaCB	4	0/20	0[a]

[a] Mortality rate not significantly different from control value ($p >> 0.05$)

Table 2. Mortality rates in chick embryos 14 days after injection of various mono-*ortho*-chlorinated chlorobiphenyls into the yolks of eggs preincubated for 4 days. A single dose (5 mg/kg) of the less toxic congeners was injected in a separate experiment

Substance injected	Dose (mg/kg egg)	Embryonic mortality	
		Ratio	%
Vehicle	–	1/20	5
2,3,3′,4,4′-pentaCB	0.1	1/20	5
	0.5	7/20	35*
	2.5	17/20	85***
2,3,3′,4,4′,5′-hexaCB	0.1	2/20	10
	0.5	6/20	30*
	2.5	18/20	90***
2,3,3′,4,4′,5-hexaCB	0.1	1/20	5
	0.5	9/20	45**
	2.5	19/20	95***
Vehicle	–	0/20	0
2,3′,4′,5-tetraCB	5	8/20	40**
2,3′,4,4′,5-pentaCB	5	9/20	45***
2,3′,4,4′,5,5′-hexaCB	5	0/20	0

* Significantly different from control ($p < 0.05$)
** Significantly different from control ($p < 0.01$)
*** Significantly different from control ($p < 0.001$)

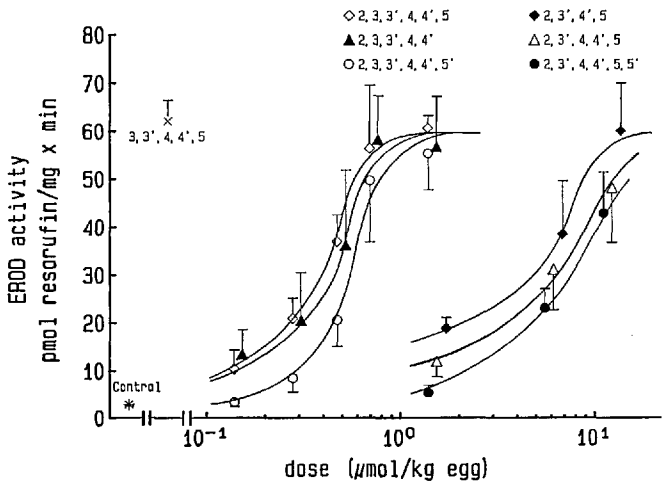

Fig. 1. Log dose-response curves for induction of hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in chick embryos by six mono-*ortho*-chlorinated chlorobiphenyls. The activity after treatment with a single dose (3.1 nmol/kg) of 3,3′,4,4′,5-pentachlorobiphenyl, assumed to cause maximal induction (Brunström and Andersson 1988), is also shown. The chlorobiphenyls (dissolved in peanut oil) were injected into the air sacs of hens' eggs preincubated for 7 days, and the EROD activity was determinated 72 h later. The dose-response curves for the coplanar chlorobiphenyls are fitted to a common maximal value. Each point represents the mean from six livers, and variation is given by the standard deviation. The controls were injected with peanut oil only, and their hepatic EROD activity (pmol resorufin/mg·min) was 3.0 ± 0.4 (mean ± SD)

were found and only a few minor hepatic lesions were detected in the embryos. Single doses (5 mg/kg) of the three less toxic congeners were injected into the eggs. Treatment with 2,3′,4′,5-tetraCB or 2,3′,4,4′,5-pentaCB caused a significant increase in embryo mortality by 2 weeks, whereas no embryos treated with 2,3′,4,4′,5,5′-hexaCB died during the experiment (Table 2). Most of the embryos exposed to 5 mg/kg 2,3′,4′,5-tetraCB or 2,3′,4,4′,5-pentaCB exhibited degenerative hepatic lesions and/or edema. In the control embryos and the embryos treated with 2,3′,4,4′,5,5′-hexaCB only a few minor hepatic lesions were noted (1/20 and 3/20, respectively).

All the tested chlorobiphenyls dose-dependently induced EROD activity in the chick embryos, with the three most toxic ones also being the strongest inducers (Fig. 1). At the highest doses tested, four of the substances induced an increase in enzyme activity to a level similar to that reached after treatment with 3.1 nmol/kg 3,3′,4,4′,5-pentaCB. By taking this degree of induction to be the maximal response to *Ah* receptor ligands (Brunström and Andersson 1988), the approximate ED_{50} values were estimated from Fig. 1 to be 0.40, 0.47, 0.56, 5.1, 6.7 and 7.8 µmol/kg for 2,3,3′,4,4′,5-hexaCB, 2,3,3′,4,4′-pentaCB, 2,3,3′,4,4′,5′-hexaCB, 2,3′,4′,5-tetraCB, 2,3′,4,4′,5-pentaCB and 2,3′,4,4′,5,5′-hexaCB, respectively.

Discussion

The mono-*ortho*-chlorinated congeners tested in the present experiment were considerably less toxic than the coplanar non-*ortho*-chlorinated ones studied previously. The lowest LD_{50} determined for the mono-*ortho*-chlorinated PCBs in the 72-h test was 4.2 µmol/kg egg for 2,3,3′,4,4′,5-hexaCB. This value is considerably higher than the corresponding LD_{50} values of 29, 9.4 and 480 nmol/kg egg previously obtained for 3,3′,4,4′-tetraCB, 3,3′,4,4′,5-pentaCB and 3,3′,4,4′,5,5′-hexaCB, respectively, in a similar test (Brunström and Andersson 1988). The difference in toxicity between the mono-*ortho*- and non-*ortho*-chlorinated congeners was about the same in the 72-h and the 2-week toxicity tests. The three most potent mono-*ortho*-chlorinated congeners maximally induced EROD activity at high doses but their ED_{50} values were about three orders of magnitude higher than that previously determined for 3,3′,4,4′,5-pentaCB (0.3 nmol/kg) (Brunström and Andersson 1988). Thus, a difference in potency of approximately three orders of magnitude between the most toxic coplanar congener (PeCB) and the mono-*ortho*-chlorinated congeners of highest potency was found in the 72-h and 2-week toxicity tests as well as in the EROD induction study.

The *Ah* receptor is present in early chick embryos (Denison et al. 1986; Brunström and Lund 1988), and the hepatic AHH activity of the early embryos is inducible by coplanar PCBs (Hamilton et al. 1983; Brunström 1986). The binding affinities of various halogenated aromatic hydrocarbons to the *Ah* receptor of C57 BL/6J mice correlate well with their potencies in inducing hepatic AHH activity in chick embryos (Poland et al. 1976). As confirmed in the present study, these compounds cause similar toxic effects in chick embryos and toxicity seems to be correlated with

binding affinity for the receptor (Rifkind et al. 1985; Brunström and Andersson 1988; Nikolaidis et al. 1988). These findings strongly suggest that the toxicity of halogenated aromatic hydrocarbons in chick embryos is mediated mainly via the *Ah* receptor.

The three mono-*ortho*-chlorinated congeners having a chloro substituent adjacent to the *ortho*-chlorine were similar in toxicity as well as in EROD-inducing potency and were about one order of magnitude more potent than the ones having a *meta*-hydrogen adjacent to the *ortho*-chlorine. Similar structure-activity relationships have also been noted in rats in vivo and in rat hepatoma cells in culture, indicating that planarity is not the only important factor determining the affinity of binding to the *Ah* receptor (Sawyer and Safe 1982; Leece et al. 1985; Parkinson and Safe 1987). The *ortho*-chlorine is buttressed by an adjacent *meta*-chlorine, and congeners having this structure should be less able to adopt a coplanar configuration than the ones with a *meta*-hydrogen adjacent to the *ortho*-chlorine. Nevertheless, the congeners having a *meta*-chlorine adjacent to the *ortho*-chlorine have a higher toxicity which is probably mediated via the *Ah* receptor.

The concentrations of mono-*ortho*-chlorinated congeners in Kanechlors are high compared with those of the non-*ortho*-chlorinated coplanar ones, and 2,3,3′,4,4′-pentaCB has been proposed to contribute as much as 3,3′,4,4′,5-pentaCB and 3,3′,4,4′-tetraCB to the "TCDD-like" toxicity of these technical PCB preparations (Kannan et al. 1988). The calclulations by Kannan et al. are based on the AHH- and EROD-inducing potencies by the various PCB congeners in rat hepatoma H-4-II-E cell lines (Sawyer and Safe 1982; Safe 1987), and on the concentrations of the congeners in the technical preparations. As an inducer of AHH and EROD in the rat hepatoma cells 3,3′,4,4′,5-pentaCB was 365 and 484 times more potent than 2,3,3′,4,4′-pentaCB, which corresponds fairly well with their relative toxicities and potencies as inducers in chick embryos. In the present study 2,3,3′,4,4′,5-hexaCB was at least as toxic and as good an EROD inducer as 2,3,3′,4,4′-pentaCB, whereas in the rat hepatoma cells it was 24 and 7 times less potent than 2,3,3′,4,4′-pentaCB at inducing AHH and EROD, respectively (Sawyer and Safe 1982). In the rat in vivo, however, 2,3,3′,4,4′,5-hexaCB was more toxic than 2,3,3′,4,4′-pentaCB, which may be due to the relatively rapid metabolism of the latter (Leece et al. 1985).

It can be concluded, that although the mono-*ortho*-chlorinated PCBs are considerably less toxic than the most potent non-*ortho*-chlorinated ones, they may still contribute to the toxicity of technical PCB mixtures owing to their relatively high concentrations in these preparations. The mono-*ortho*-chlorine-substituted PCB congeners differ in toxicity, with the ones having a chloro substituent in the *meta* position adjacent to the *ortho*-chlorine being more potent than those having a hydrogen in this position. Further studies on non-*ortho*- and mono-*ortho*-chlorinated congeners in biological samples are needed to evaluate their contribution to the environmental hazards of PCBs.

Acknowledgements. Thanks are due to Dr Åke Bergman for supplying the PCBs and to Katarina Hjelm for skilful technical assistance. This study was supported by the Swedish Natural Science Research Council (No. B-TF 9089-300) and the National Swedish Environmental Protection Board (No. 5326239-0).

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